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# Impacts of Simulated N Deposition on Plants and Mycorrhizae from Spanish Semiarid Mediterranean Shrublands

Raúl Ochoa-Hueso,[1,2]* M. Esther Pérez-Corona,[3] and Esteban Manrique[1]

*[1]Museo Nacional de Ciencias Naturales, Consejo Superior de Investigaciones Científicas (MNCN-CSIC), C/Serrano 115 dpdo., 28006 Madrid, Spain; [2]Área de Biodiversidad y Conservación, Departamento de Biología y Geología, Universidad Rey Juan Carlos, C/Tulipán s/n., 28933 Móstoles, Spain; [3]Department of Ecology, Faculty of Biology, Universidad Complutense de Madrid, C/José Antonio Novais 2, 28040 Madrid, Spain*

## ABSTRACT

Nitrogen (N) deposition threatens European Mediterranean ecosystems but investigation and understanding of impacts are limited. We report plant responses from an ongoing field N fertilization experiment conducted in a kermes oak shrubland, where $NH_4NO_3$ has been added for 1.5 years at four rates (0, 10, 20, and 50 kg N $ha^{-1}$ $y^{-1}$). Two annual plants (*Asterolinon linum-stellatum* and *Limonium echiodes*) were negatively affected by N fertilization in terms of density and growth. However, responses were only evident when accounting for between-plot differences in soil $NO_3^-$-N and $NH_4^+$-N. Responses of *A. linum-stellatum* to simulated N deposition were also dependent on microhabitat, with the most negative effects found in the interspaces between rosemary shrubs. Negative effects were attributed either to increased soil $NH_4^+$-N or to a nutritional (N to P) imbalance. Mycorrhizal infection rates were not altered by N addition in the case of *L. echioides*, whereas mycorrhizal colonization of *A. linum-stellatum* roots increased with N in those individuals growing under shrub protection. Living cover of rosemary shrubs was also reduced by simulated N deposition as a consequence of a reduced interannual twig growth. Contrary to annual plants, tissue N content and C:N ratios in rosemary were not affected by simulated N deposition. Overall, our data suggest a high sensitivity of plant communities from moderately polluted semiarid Mediterranean shrublands to N deposition, highlight the role of different forms of inorganic N on plant response to N deposition, and support the importance of conducting similar experiments in other Mediterranean areas spanning a wide range of climatic, soil, and background N deposition conditions.

**Key words:** environmental heterogeneity; experimental fertilization; inoculum; microambient; nutrient P imbalance; soil N toxicity; vascular plants.

## INTRODUCTION

It is urgent to evaluate the sensitivity of terrestrial ecosystems to disturbance to understand the implications of human activities on the global-scale biodiversity loss (Sala and others 2000). One such disturbance is anthropogenic nitrogen (N) deposition

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**Corresponding author; e-mail:* raul.ochoa@mncn.csic.es

that has rarely been studied in European semiarid Mediterranean ecosystems (Bobbink and others 2010; Ochoa-Hueso and others 2011). However, N deposition is considered a potential threat to the region (Fenn and others 2003) and also predicted to increase in the future (Phoenix and others 2006). In water-limited semiarid Mediterranean ecosystems, N as a pollutant accumulates during the summer; dry deposits become available as pulses at the end of the seasonal drought coinciding with the autumnal rains (Allen and others 2009) and the ability of plants to capture N from these brief pulses is expected to influence plant growth, survival, and competitive ability (James and Richards 2005).

Given the lack of information concerning other Mediterranean areas, Californian chaparral and coastal sage scrub are usually viewed as models for predicting potential effects of enhanced anthropogenic-related N inputs on Mediterranean ecosystems worldwide (Ochoa-Hueso and others 2011). These regions are all primarily water-limited and secondarily N-regulated and limited, whereas phosphorus (P) also plays an important role in the Mediterranean (Hobbs and others 1995), regulating responses to N enrichment at the global scale (Vitousek and others 2010; Ochoa-Hueso and others 2011).

In parallel field experiments conducted in Californian chaparral and coastal sage scrub ecosystems, Vourlitis and Pasquini (2009) found that an alteration of the plant community after 5 years of 50 kg N $ha^{-1}$ $y^{-1}$ addition only occurred in the coastal sage scrub. The main response was in terms of decreased shrub biomass, whereas no change in ephemeral species biomass was reported in the same study. In contrast, Witkowski (1989a) reported an increase of ephemeral species biomass as the main impact of N fertilization in a lowland fynbos and highlighted that those N effects were only evident when accounting for the initial heterogeneity of the plant cover on the experimental plots. Allen and others (2009) have related N pollution to exotic grass invasion in Californian deserts and chaparral, with a resultant displacement of native annual forbs. Bonanomi and others (2006) have also found that N enrichment strongly increases the living biomass, while it maintains very low species diversity in Mediterranean semi-natural grasslands, and they warned about the potential threat of the co-occurrence of N enrichment and land abandonment for the maintenance of high species diversity in these ecosystems. Other well-known effects of N addition in Mediterranean ecosystems are increased leaching of inorganic N (Vourlitis and others 2009) and organic N and carbon (C) (Smemo and others 2006) and also altered litter and plant tissue C:N ratios (Witkowski 1989b; Vourlitis and others 2009).

The mycorrhizal feedback hypothesis states that composition and density of mycorrhizal fungal communities (that is, soil inoculum quality and quantity) may influence composition of plant communities, predicting that N enrichment may generate reciprocal changes of plants and arbuscular mycorrhizal fungi (AMF) by making N more accessible to plant roots (Johnson and others 2008). Furthermore, the functional equilibrium model predicts that addition of soil nutrients would reduce allocation to roots and, depending on the P status of soils, to mycorrhizae (Johnson and others 2008). Thus, species typical of oligotrophic environments relying on mycorrhizal symbiosis and with little ability to shift biomass allocation from below- to aboveground structures will be displaced by species with less mycorrhizal dependency, investing fewer resources on mycorrhizae and characterized by a more plastic biomass allocation capacity.

In arid and semiarid regions, shrubs are often considered nurse plants to the annual plant community, being islands of fertility in harsh environments as a result of organic matter inputs and a more active nutrient cycling. Other benefits provided by shrubs are sheltering from direct radiation and wind desiccation, moisture retention, and temperature amelioration (Pugnaire and others 2004). Soil fertility and moisture can affect net competitive outcome between different plant functional groups (Van der Waal and others 2009), making it important to measure how plant interactions and spatial aggregation shift along environmental gradients (Freckleton and others 2009). Debate exists on whether the stress-gradient hypothesis (predicting a shift from facilitative to competitive interactions with environmental stress amelioration) fits all plant–plant interactions and abiotic stress conditions (Bertness and Callaway 1994; Maestre and others 2006). If it does not, competition could also dominate at both extremes of stress gradients (Maestre and Cortina 2004; Maestre and others 2006). For this reason, understanding how facilitation and competition (shrub–shrub, shrub–annual, or annual–annual) are modified by abiotic conditions is important to predict impacts of atmospheric N deposition. This knowledge will also be relevant for developing tools aiming to enhance ecosystem restoration (Brooker and others 2008).

The main hypotheses of this study are: (i) N addition to a semiarid Mediterranean shrubland will increase plant biomass production of individual species (Bonanomi and others 2006), unless a critical threshold is exceeded (Ochoa-Hueso and Manrique 2010); (ii) mycorrhizal responses to N enrichment will depend on the N:P status (Egerton-Warburton and others 2007) and/or ammonium ($NH_4^+$-N) availability (Yoshida and Allen 2001) of soils and on the potential inoculum quality and quantity (Johnson and others 2008); (iii) plant tissue N content will increase independently of the effects of N fertilization on plant productivity; (iv) the interaction between annual plants and shrubs (annual plants acting as commensal and shrubs either playing a facilitative or competitive role) will be altered by the artificial gradient imposed by the experimental N fertilization; (v) plant responses will depend on the spatial distribution of pre-existing and superimposed gradients in soil resources (Witkowski 1989a; Cruz and others 2003), mainly in soil nitrate ($NO_3^-$-N), the dominant form of mineral N in semiarid shrublands from central Spain, and soil pH (Ochoa-Hueso and Manrique 2011).

# METHODS

## Study Area

This study was conducted in central Spain (Nature Reserve El Regajal-Mar de Ontígola, 40°00′N, 3°36′W; mean altitude ~500–600 m asl), within a kermes oak (*Quercus coccifera* L.) shrubland (Figure S1). The climate of the area is semiarid Mediterranean, meaning that winter is cold and wet and summer is warm and dry. Total rainfall amount is approximately 425 mm $y^{-1}$, predominantly falling between October and May (Rivas-Martínez 1987). The vegetation of this shrubland is characterized by dense thickets of kermes oak, with interspaces between these thickets that are dominated by sclerophyllous shrubs, short-lived therophytes during the spring growing season and a diverse and well-developed biological soil crust (Figure S1; Crespo and Barreno 1975). Among the dominant shrub species are rosemary (*Rosmarinus officinalis* L.—Labiatae; mean cover (±SE) in the early spring 2009 = 35.9% ± 2.1), *Lithodora fruticosa* (L.) Griseb. and *Rhamnus lycioides* L.. Among the herbaceous species are *Asterolinon linum-stellatum* (L.) Duby, *Limonium echiodes* L. (Mill.) (Plumbaginaceae), *Stoibrax dichotomum* (L.) Rafin. (Umbelliferae), *Iberis crenata* Lam. (Cruciferae) or *Platycapnos spicata* (L.) Bernh. There are no leguminous shrubs and herbs at the study site. The shrubland is mainly located at the top and middle parts of hills separated by small gullies. Soils are quaternary limestone and $NO_3^-$-N (mean [mg·kg of soil$^{-1}$] in winter 2008/09 ± SE = 8.88 ± 1.10) is the dominant form of inorganic N over $NH_4^+$-N [mean (mg kg of soil$^{-1}$) in winter 2008/09 ± SE = 5.94 ± 0.65], as it corresponds to a basic soil pH (mean in winter 2008/09 ± SE = 8.01 ± 0.08). Soil organic matter [mean (%) in winter 2008/09 ± SE = 5.37 ± 0.45], organic N [mean (%) in winter 2008/09 ± SE = 0.26 ± 0.02], C:N (mean in winter 2008/09 ± SE = 12.19 ± 0.34) and $P_2O_5$-P [mean (mg·kg of soil$^{-1}$) in winter 2008/09 ± SE = 0.85 ± 0.60] values are within the description of a typical semiarid Mediterranean soil.

## Nitrogen Fertilization Experiment

In October 2007, six randomized blocks with four plots each were established within open areas of the kermes oak shrubland dominated by rosemary shrubs. Each plot (2.5 × 2.5 m) was separated from the others within the block by two perpendicular crossed 1-m buffer bands. Each plot was randomly assigned to one of the following treatments over the background N deposition: 0, 10, 20, and 50 kg N $ha^{-1}$ $y^{-1}$. Background N deposition was estimated at approximately 6.1 kg N $ha^{-1}$ $y^{-1}$, estimation based on the CHIMERE model (Vivanco and others 2009). All of these treatments excluding the latter either fell within the predicted 2,050 deposition scenarios for the Mediterranean Basin or are similar to N deposition loads measured in certain Mediterranean areas of California (Phoenix and others 2006; Fenn and others 2003).

Nitrogen was applied monthly with a hand sprayer in 2 l doses of an aqueous solution containing ammonium nitrate ($NH_4NO_3$). In the case of the control plots, we only added water. The use of a 1:1 proportion of oxidized and reduced N is in agreement with rain chemistry measurements at the study site (Ochoa-Hueso and others 2013). During the driest period (July–August), fertilization was not done and a 3-month total N load was applied in September to simulate the peak of N availability with the onset of rains (Fenn and others 2003). Nitrogen was applied to the whole surface of the plots, including soil surface, biological soil crusts and plants. Our experimental N addition (dissolved in water) only gently and slightly rewetted the soil surface, with manipulated soils becoming dry in a few minutes (personal observation). This allows added N to accumulate in the first mm of soil and thus we consider this experimental manipulation as suitable to simulate N deposition in these ecosystems, even when assuming differences with real dry N deposition. The area is also close to a main river and morning dew frequently moistens the soil.

## Annual Plant Survey

*Asterolinon linum-stellatum* (L.) Duby (Primulaceae) and *L. echiodes* (L.) Mill. (Plumbaginaceae) surveys were conducted in March 2009, approximately 1.5 years after the establishment of the field experiment. These species were chosen for sampling purposes among all the annual species present at the study site because they were present across most of the experimental plots, whereas the rest of the annual species were too random/scarce for subsequent statistical comparisons/analyses.

Each plot was gridded into twelve 0.5 × 0.5 m squares, leaving a perimeter band of 0.5 m on the N and S sides, and 0.25 m on the E and W sides (Figure S1c). Six squares in alternating position were sampled. Each square was also divided into twenty-five 10 × 10 cm sub-squares, where density counts were made, followed by shoot and root harvests (see below). Shrub cover was also recorded as presence or absence for each sub-square and then transformed to percentage for the whole plot.

Six to twenty individuals of *A. linum-stellatum* and *L. echioides* (when present) were harvested from each plot. Given that the individuals of both species had low biomass production and the small amount of the plants harvested, our sampling had no disturbance effect on the experimental plots. Once in the lab, plants were oven dried at 65°C to constant weight and then weighed. In the case of *A. linum-stellatum*, individual plants were surveyed both in the interspaces and under shrubs, and shoots and roots were also weighed separately to calculate root:shoot ratios. Given the small size of the individuals and the relatively thin and shallow roots, we could be sure of harvesting the whole root system in all cases. In contrast, we only sampled aboveground biomass for *L. echioides* given the difficulty of obtaining complete root systems. In addition, this species was never present under shrubs, so we only sampled individuals in the interspaces.

The spatial aggregation index ($I_a$) of *A. linum-stellatum* was assessed using the spatial analysis by distance indices (SADIE) provided by the SADIE-Shell program (Perry and others 1999). This parameter gives information on the overall spatial pattern in the data set; a clumped spatial pattern being indicated by $I_a$ greater than 1, random pattern $I_a$ approximately equal to 1 and regular pattern by $I_a$ less than 1 (Maestre 2003); $I_a$ was only calculated for plants growing in the interspaces. SADIE analyses were conducted separately for each plot using 6,009 permutations. The relative neighbor effect (RNE) index was also calculated to account for the effect of rosemary shrubs (neutral, facilitative, or competitive) on *A. linum-stellatum*. RNE was calculated as follows (Maestre and Cortina 2004),

$$\mathrm{RNE} = (I - S)/M, \tag{1}$$

where $I$ and $S$ are plant variables at interspaces or under shrub cover, respectively, and $M$ can be either $I$ (when $I > S$) or $S$ (when $S > I$).

## Arbuscular Mycorrhizal Fungi

For AMF determinations, dry roots of *A. linum-stellatum* and *L. echioides* were re-hydrated, cleared with 10% KOH and stained with trypan blue (0.05% in a 2:1:1 lactic acid:glycerol:distilled water solution). The modified line intersection method was used to determine the percentage of root length colonized by AMF (McGonigle and others 1990) and the presence of hyphae, vesicles, and arbuscules was scored. Determinations were also separately made for *A. linum-stellatum* living in the interspaces or under shrubs and for *L. echioides* in the interspaces. Given the difficulty in obtaining complete roots for *L. echioides*, we did AMF determinations on incomplete root systems, which could have conditioned our results (see below).

## Rosemary Survey

At the beginning of 2008 and 2009 growing seasons (~mid February), 10 twigs of five rosemary shrubs per plot were marked at 1 cm from the tip. At the end of each growing season (~mid July), marked twigs were harvested, oven dried at 65°C to constant weight and then weighed. Total rosemary shrub cover was also estimated for each plot at the end of each season by measuring the maximum and the perpendicular diameters of each individual present in the plot and summing the area covered by all individuals.

## Tissue Chemistry

Tissue C and N content (%) was analyzed in complete shoots of *A. linum-stellatum* and *L. echioides* and in leaves of *R. officinalis* with a CHN Perkin Elmer 2400 elemental analyzer.

## Soil Sampling and Chemical and Physical Analyses

Eight soil cores 0–4 cm depth were randomly taken in the external band of each plot just before (winter 2008) and during (spring 2009) the 2009 growing season. Bulked samples were stored in cool conditions until returned to the lab, where they were air-dried, thoroughly homogenized and 2-mm sieved prior to analyses. Soil pH (in water), organic matter, organic and inorganic ($NO_3^-$-N and $NH_4^-$-N) N, organic C, total and extractable P, and extractable base (Ca, Na, Mg, and K) and non-base cations (Fe, Mn, Zn, Cu, and Al) were all analyzed at the Unit of Analysis of the Centre for Environmental Sciences (CSIC, Madrid, Spain) as described in Campos-Herrera and others (2008) and Ochoa-Hueso and Manrique (2011). Soil water content was also calculated as the difference (in % weight change) between a certain amount of fresh soil and the same amount after oven-drying at 65°C to constant weight.

## Statistical Analyses

Prior to any analysis, data sets were checked for normality assumptions and, when not met, data were appropriately transformed as indicated. Data were also transformed when required to reduce data variance.

*Asterolinon linum-stellatum* responsiveness was analyzed with two-way multivariate analyses of the variance (two-way MANOVA). Response variables were plant density, shoot and root biomass per individual, and root:shoot ratios. Wilks' $\lambda$ was used for testing significant effects. Micro-site (interspace vs. shrub cover) and N supply were the fixed factors. We also tested for the effects of soil covariates (from both winter and spring surveys) by covariance analyses (two-way MANCOVA). Nitrogen fertilization effects on the aggregation index and on the RNE for *A. linum-stellatum* density, shoot and root biomass per individual, and root:shoot ratios were also evaluated by MANOVA and MANCOVA procedures as described above. For these analyses, shoot and root biomass per individual data were $\log_{10}(x + 1)$-transformed to meet the test's assumptions. Because it was never present under shrubs, *L. echioides* density and biomass per individual were analyzed by one-way ANOVA and ANCOVA with N as fixed factor.

AMF colonization (% root infected and % arbuscules) of *A. linum-stellatum* roots was analyzed by means of two-way ANOVA and also independently analyzed for N effects on plants under shrubs or at interspaces. One-way ANOVAs were also used for the AMF colonization of *L.* echioides's roots. Tissue chemistry (C, N, and C:N ratio) of different species was evaluated by means of one-way (*L. echiodes* and *R. officinalis*) or two-way (*A. linum-stellatum*) ANOVAs.

Plant growth variables, $I_a$, RNE and mycorrhizae of *A. linum-stelatum* and *L. echioides* were related to soil variables independently of the N treatments by the use of stepwise linear regressions. Plant growth variables were also related to % AMF and arbuscules for these two species, distinguishing by micro-site in the case of *A. linum-stelatum*.

Effects of N fertilization on rosemary cover (log-transformed) and rosemary annual twig growth were analyzed as the change from summer 2008 to summer 2009 by the means of one-way ANOVAs. Covariance analyses were also used when appropriate to improve statistical outputs.

The covariates used for the analyses on annual plants and mycorrhizae were always from winter 2008 because they showed stronger relationships with plant traits. In the case of rosemary, we tested for the covariance of four-season averaged data and also for the standard deviation of data, assumed to represent nutrient seasonality to a certain degree (Ochoa-Hueso and Manrique 2011).

Post-hoc multiple comparisons were made with LSD tests and statistical significance was considered at the $P \leq 0.05$ level. SPSS17 was used for all statistical analyses.

# RESULTS

When soil $NO_3^-$-N and $NH_4^+$-N (log-transformed) were jointly used as covariates, we found an overall significant and negative effect of N fertilization on *A. linum-stellatum*. Shrubs also determined *A. linum-stellatum* micro-distribution and morphology; that is, there was higher density, smaller plant size and higher root:shoot ratios under shrubs (Table 1, Figure 1).

Similarly, when soil $NO_3^-$-N and pH were jointly used as covariates, there was an overall effect of N fertilization on the spatial distribution pattern ($I_a$) and the interaction (RNE) between *A. linum-stellatum* and rosemary shrubs. This significant effect was largely due to the 50 kg N $ha^{-1}$ $y^{-1}$ treatment (Table 2, Figure 2).

Using soil $NO_3^-$-N as a covariate resulted in a significant and dose-related decrease of *L. echioides* density (log-transformed) with N fertilization (df = 3; $F = 6.48$; $P < 0.01$; Figure 3), whereas aboveground biomass production per individual was not significantly affected by N fertilization (df = 3; $F = 1.24$; $P = 0.33$; mean aboveground biomass (g) ± SE = 0.02 ± 0.00).

Arbuscular mycorrhizal colonization and % arbuscules (mean ± SE = 19.16 ± 1.72) were not affected by simulated N deposition; however, AMF colonization was significantly increased by N under shrubs when using $NH_4^+$-N as a covariate (df = 3; $F = 3.27$; $P = 0.04$; Table 1, Figure 4). Aboveground productivity of *A. linum-stellatum* individuals living under

**Table 1.** Nitrogen Fertilization and Micro-site Effects on *A. linum-stellatum*'s Growth-related Variables and Mycorrhizae

| | MANOVA | | | Density (ind m$^{-2}$) | | | Shoot biomass (g) ind$^{-1}$ | | | Root biomass (g) ind$^{-1}$ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | df | *F* | *P* | df | *F* | *P* | df | *F* | *P* | df | *F* | *P* |
| Nitrogen | 12 | 1.09 | 0.38 | 3 | 1.58 | 0.21 | 3 | 0.51 | 0.68 | 3 | 0.82 | 0.49 |
| Micro-site | 4 | 10.44 | $<$**0.01** | 1 | 11.23 | $<$**0.01** | 1 | 27.27 | $<$**0.01** | 1 | 8.26 | **0.01** |
| N × micro-site | 12 | 0.39 | 0.96 | 3 | 0.09 | 0.96 | 3 | 0.05 | 0.99 | 3 | 0.03 | 0.99 |

| | Root:shoot | | | MANOVA | | | % AMF | | | % Arbuscules | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | df | *F* | *P* | df | *F* | *P* | df | *F* | *P* | df | *F* | *P* |
| Nitrogen | 3 | 1.42 | 0.25 | 6 | 1.06 | 0.40 | 3 | 0.32 | 0.81 | 3 | 1.54 | 0.22 |
| Micro-site | 1 | 18.90 | $<$**0.01** | 2 | 0.42 | 0.66 | 1 | 0.61 | 0.44 | 1 | 0.81 | 0.44 |
| N × micro-site | 3 | 0.37 | 0.77 | 6 | 1.06 | 0.39 | 3 | 1.55 | 0.21 | 3 | 0.33 | 0.80 |

| | MANCOVA[1] | | | Density (ind m$^{-2}$) | | | Shoot biomass (g) ind$^{-1}$ | | | Root biomass (g) ind$^{-1}$ | | | Root:shoot | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | df | *F* | *P* | df | *F* | *P* | df | *F* | *P* | df | *F* | *P* | df | *F* | *P* |
| Nitrogen | 12 | 2.02 | **0.03** | 3 | 2.84 | **0.05** | 3 | 0.37 | 0.77 | 3 | 3.05 | **0.04** | 3 | 1.67 | 0.19 |
| Micro-site | 4 | 10.18 | $<$**0.01** | 1 | 14.71 | $<$**0.01** | 1 | 27.40 | $<$**0.01** | 1 | 10.29 | $<$**0.01** | 1 | 19.67 | $<$**0.01** |
| N × micro-site | 12 | 0.41 | 0.96 | 3 | 0.12 | 0.95 | 3 | 0.05 | 0.98 | 3 | 0.04 | 0.99 | 3 | 0.39 | 0.76 |

*Significant effects ($P < 0.05$) are in bold. df degrees of freedom.*

[1] *Soil nitrate and soil ammonium (log-transformed).*

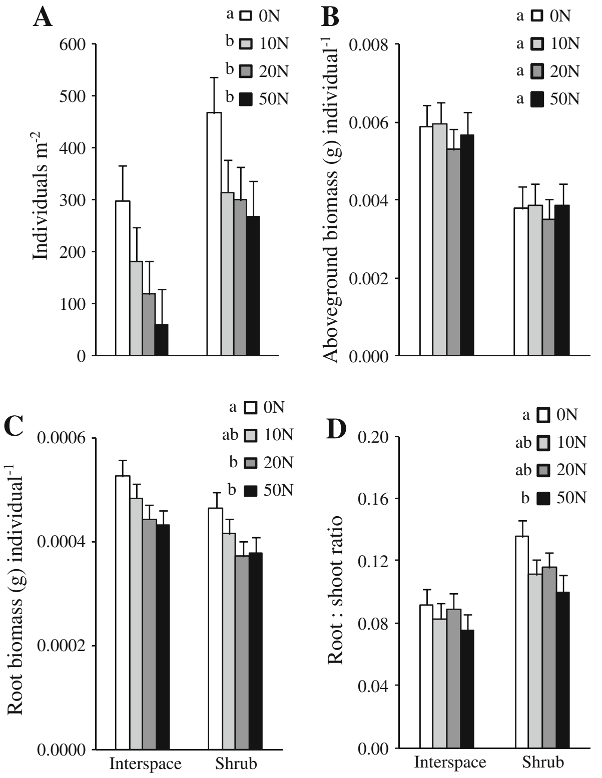

**Figure 1.** Nitrogen fertilization and micro-site effects on *A. linum-stellatum*'s growth-related variables. Data are shown after using soil nitrate and soil ammonium (log-transformed) as covariates. *Different letters* between treatments for each variable indicates significant ($P < 0.05$) differences after LSD tests. For significant effects between micro-sites see Table 1. *White* (0 N), *light-grey* (10 N), *dark-grey* (20 N), *black* (50 N). *Bars* are SE.

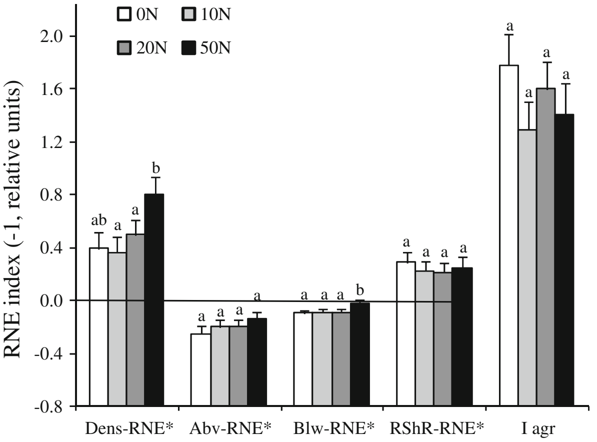

**Figure 2.** Nitrogen fertilization effects on the relative neighbor effect (RNE) of shrubs on *A. linum-stellatum*. Effects on the spatial distribution pattern $I_a$ (no units) of *A. linum-stellatum* are also presented. Data are shown after using soil nitrate and pH as covariates. *Different letters* between treatments for each variable indicates significant ($P < 0.05$) differences after LSD tests. *Dens* plant density, *abv* aboveground biomass, *blw* belowground biomass, *RShR* root to shoot ratio, *I agr* aggregation index. *White* (0 N), *light-grey* (10 N), *dark-grey* (20 N), *black* (50 N). *Bars* are SE.

shrubs, but not in the interspaces, was positively correlated to AMF colonization ($R^2 = 0.16$; $P = 0.06$) and % arbuscules ($R^2 = 0.21$; $P = 0.02$), respectively. The rest of the plant growth variables were unrelated to mycorrhizae (data not shown). AMF colonization rates of *L. echioides* (df = 3; $F = 0.13$; $P = 0.94$) were not significantly altered by N addition (mean % infection ± SE = 14.78 ± 2.83).

Nitrogen fertilization increased tissue N content in *A. linum-stellatum* (df = 3; $F = 9.65$; $P < 0.01$) and *L. echioides* (df = 3; $F = 6.28$; $P < 0.01$) but not in rosemary (df = 3; $F = 0.78$; $P = 0.52$). There was also a significant decrease of tissue C:N ratios in *A. linum-stellatum* (df = 3; $F = 8.95$; $P < 0.01$) and *L. echioides* (df = 3; $F = 7.14$; $P < 0.01$) associated with N fertilization. These changes only occurred with the highest N treatment (Figure 5). The N × micro-site interaction was not significant for tissue N (df = 3; $F = 0.21$; $P = 0.89$) and CN (df = 3; $F = 0.33$; $P = 0.81$) in the case of *A. linum-stellatum*,

**Table 2.** Nitrogen Fertilization Effects on the Relative Neighbor Effect (RNE) of Shrubs on *A. linum-stellatum*

| | MANOVA | | | Dens-RNE | | | Abv-RNE | | | Blw-RNE | | | RShR-RNE | | | *I* agr | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | df | *F* | *P* | df | *F* | *P* | df | *F* | *P* | df | *F* | *P* | df | *F* | *P* | df | *F* | *P* |
| Nitrogen | 1 | 1.18 | 0.32 | 3 | 1.18 | 0.34 | 3 | 0.20 | 0.89 | 3 | 0.14 | 0.93 | 3 | 0.37 | 0.78 | 3 | 1.45 | 0.26 |
| | MANCOVA[1] | | | Dens-RNE | | | Abv-RNE | | | Blw-RNE | | | RShR-RNE | | | *I* agr | | |
| | df | *F* | *P* | df | *F* | *P* | df | *F* | *P* | df | *F* | *P* | df | *F* | *P* | df | *F* | *P* |
| Nitrogen | 15 | 2.25 | **0.02** | 3 | 2.17 | 0.13 | 3 | 0.64 | 0.60 | 3 | 2.35 | 0.11 | 3 | 0.22 | 0.88 | 3 | 1.07 | 0.39 |

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*Effects on the spatial distribution pattern of A. linum-stellatum are also presented. Significant effects ($P < 0.05$) are in bold. df degrees of freedom, dens plant density, abv aboveground biomass, blw belowground biomass, RShR root:shoot ratio, I agr aggregation index.*

[1] *Soil nitrate and soil pH.*

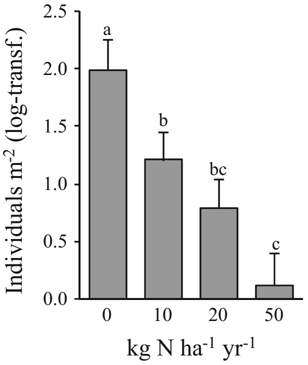

**Figure 3.** Nitrogen fertilization effects on *L. echioides* density (individuals m$^{-2}$). Data are shown after using soil nitrate as a covariate. *Different letters* between N treatments indicates significant ($P < 0.05$) differences after LSD tests. *Bars* are SE.

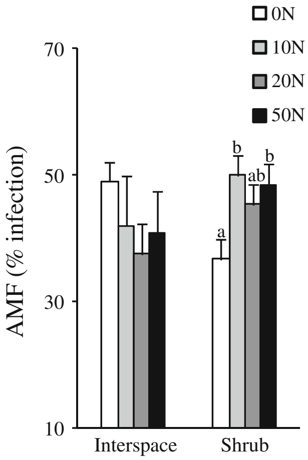

**Figure 4.** Nitrogen fertilization and micro-site effects on *A. linum-stellatum*'s AMF colonization. % AMF under shrubs data are presented using soil ammonium as a covariate. *Different letters* between treatments for each variable within each micro-site indicates significant ($P < 0.05$) difference after LSD tests. For significance between micro-sites, see Table 1. *White* (0 N), *light-grey* (10 N), *dark-grey* (20 N), *black* (50 N). *Bars* are SE.

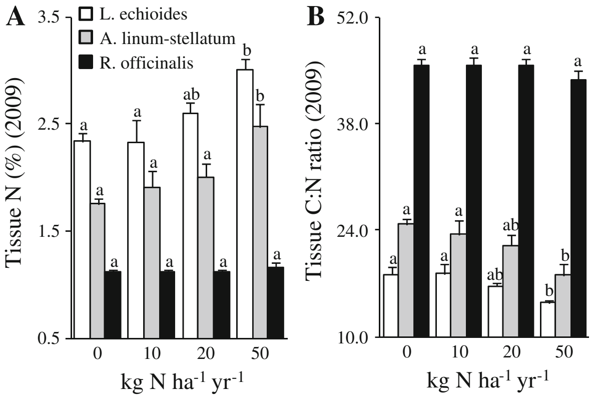

**Figure 5.** Nitrogen fertilization effects on tissue chemistry of *Asterolinon linum-stellatum*, *Limonium echioides*, and *Rosmarinus officinalis*. *Different letters* between N treatments indicates significant ($P < 0.05$) differences after LSD tests. *Bars* are SE.

whereas the micro-site effect was always significant (N: df = 3; $F = 4.51$; $P = 0.04$); (CN: df = 3; $F = 5.35$; $P < 0.01$). Tissue N and CN were consistently higher and lower, respectively, under shrub cover (data not shown). Tissue C content was not altered by N deposition nor by micro-site in any of the species analyzed (data not shown).

Stepwise linear regressions showed that soil $NH_4^+$-N was negatively related to *A. linum-stellatum* aboveground biomass in the interspaces and to *A. linum-stellatum* AMF under shrubs (Table 3), which has important implications in terms of *A. linum-stellatum* response to simulated N deposition. In addition, soil C:N ratio was negatively related to *A. linum-stellatum* root:shoot ratio in the interspaces; i.e., the higher the proportion of soil N, the higher the proportion of roots. Other essential macro- and micro-nutrients and soil texture were also related to different variables concerning *A. linum-linum stellatum* or *L. echioides*. For example, Ca availability played a major role regulating competitive/facilitative plant–plant interactions (i.e., RNE) for *A. linum-stellatum*; Zn and sand percentage were positively related to *A. linum-linum stellatum* root growth in the interspaces and under shrubs, respectively; Fe was negatively correlated with *A. linum-linum stellatum* density under shrubs; and Na controlled *A. linum-linum stellatum* root:shoot ratio in the interspaces and *L. echioides* density.

Rosemary cover, measured as the log-transformed difference between cover from 2008 and 2009, was significantly reduced by N fertilization (df = 3, 20; $F = 3.34$; $P = 0.04$; Figure 6A). Multiple comparisons showed that there were significant differences between the control and the 20 and 50 kg N ha$^{-1}$ y$^{-1}$ treatments. Twig growth, also evaluated as the difference between 2008 and 2009, was significantly affected by N fertilization when $NO_3^-$-N seasonality was included in the analysis as a covariate (df = 3, 16; $F = 3.14$; $P = 0.05$; Figure 6B).

## Discussion

In this study, we have shown that growth and cover of a woody plant as well as of two annual plants were negatively affected by simulated N deposition. The asymmetric interaction between

**Table 3.** Significant ($P < 0.05$) Soil Variables Related to *A. linum-stellatum* and *L. echioides* After Stepwise Linear Regressions

| | Var. | Sign | $R^2$ | $P$ | Var. | Sign | $R^2$ | $P$ | Var. | Sign | $R^2$ | $P$ | Var. | Sign | $R^2$ | $P$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Asterolinon linum-stellatum* | | | | | | | | | | | | | | | | |
| Interspace | | | | | | | | | | | | | | | | |
| Ind $m^{-2}$ | $NH_4^+$ | – | 0.18 | 0.04 | Mg | + | 0.34 | 0.01 | NS | | | | | | | |
| Shoot (g) $ind^{-1}$ | NS | | | | NS | | | | NS | | | | | | | |
| Root (g) $ind^{-1}$ | Zn | + | 0.45 | <0.01 | $NH_4^+$ | – | 0.59 | <0.01 | NS | | | | | | | |
| Root:shoot | C:N | – | 0.22 | 0.02 | Cu | + | 0.49 | <0.01 | NS | | | | | | | |
| AMF (%) | NS | | | | NS | | | | NS | | | | | | | |
| Arbuscules (%) | NS | | | | NS | | | | NS | | | | | | | |
| Shrub | | | | | | | | | | | | | | | | |
| Ind $m^{-2}$ | Fe | – | 0.21 | 0.03 | NS | | | | NS | | | | | | | |
| Shoot (g) $ind^{-1}$ | Ca | – | 0.27 | 0.01 | NS | | | | NS | | | | | | | |
| Root (g) $ind^{-1}$ | % sand | + | 0.44 | <0.01 | NS | | | | NS | | | | | | | |
| Root:shoot | Na | + | 0.20 | 0.03 | NS | | | | NS | | | | | | | |
| AMF (%) | $NH_4^+$ | – | 0.38 | <0.01 | Ca:Al | + | 0.49 | <0.01 | % clay | – | 0.63 | <0.01 | Fe | + | 0.72 | <0.01 |
| Arbuscules (%) | NS | | | | NS | | | | NS | | | | | | | |
| RNE | | | | | | | | | | | | | | | | |
| Ind $m^{-2}$ | NS | | | | NS | | | | NS | | | | | | | |
| Shoot (g) $ind^{-1}$ | Ca | – | 0.40 | <0.01 | NS | | | | NS | | | | | | | |
| Root (g) $ind^{-1}$ | Ca | – | 0.69 | <0.01 | Na | + | 0.86 | <0.01 | Mg | – | 0.79 | <0.01 | | | | |
| Root:shoot | NS | | | | NS | | | | NS | | | | | | | |
| *I* agr | NS | | | | NS | | | | NS | | | | | | | |
| *Limonium echioides* | | | | | | | | | | | | | | | | |
| Ind $m^{-2}$ | Na | + | 0.64 | <0.01 | Al | – | 0.78 | <0.01 | Mg | + | 0.82 | <0.01 | | | | |
| Shoot (g) $ind^{-1}$ | NS | | | | NS | | | | NS | | | | | | | |
| AMF (%) | NS | | | | NS | | | | NS | | | | | | | |

*''Interspace'' and ''shrub'' denote micro-site. RNE relative neighbor effect index, var. selected variable, sign type of effect (+ or −), NS none variable selected.*

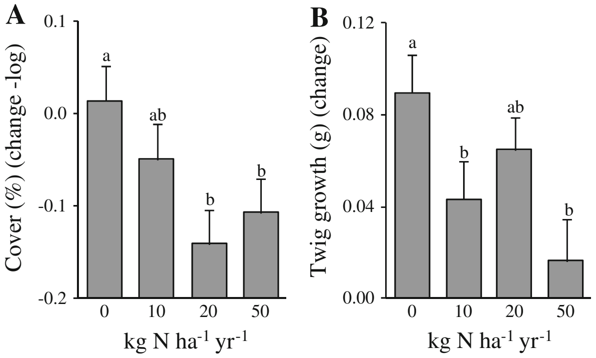

**Figure 6.** Nitrogen fertilization effects on rosemary (**A**) cover (change between 2009 and 2008), and (**B**) twig growth (change between 2009 and 2008). Data for cover are log-transformed. Data are shown after using soil nitrate (**A**) and nitrate seasonality (**B**) as covariates. *Different letters* between treatments for each variable indicates significant ($P < 0.05$) difference after LSD tests. *Bars* are SE.

*A. linum-stellatum* and shrubs was also altered. However, all these effects were only evident when accounting for pre-existing and superimposed environmental gradients in soil $NO_3^-$-N, $NH_4^-$-N, and pH, the former the most abundant form of inorganic N in the study site (Ochoa-Hueso and Manrique 2011). This highlights the importance of the environmental heterogeneity in terms of resource availability, soil properties, and micro-site distribution in highly spatial and temporal heterogeneous ecosystems to detect significant alterations in the context of field global change experiments. Other authors studying field fertilization effects on the natural vegetation have highlighted that responses can be initially masked by environmental heterogeneity and that covariance analyses help significantly in overcoming this problem (Witkowski 1989a, b). Similar experiments in other semiarid Mediterranean-type ecosystems (coastal sage scrub) have found significant effects of N additions on the woody plant community but did not detect any alteration of the annual community in terms of biomass or abundance of certain species (chaparral, coastal sage scrub), even after 5 years of continuous N fertilization (Vourlitis and Pasquini 2009). In contrast, Witkowski (1989a) demonstrated that responses to N fertilization in a South-African fynbos were mostly associated with the herbaceous plants.

*Asterolinon linum-stellatum* and *L. echioides* densities were reduced by N fertilization and it is well known that seed emergence can be either positively or negatively influenced by soil $NO_3^-$-N or $NH_4^+$-N (Rashid and others 2007). Similar to our results, Pérez-Fernández and Rodríguez-Echevarría (2003) related the negative effects of nitrogenous compounds on seed germination to specific abilities to colonize low-N soils. Ochoa-Hueso and Manrique (2010) found an increased overall germination rate of a seed bank from the same study site in Aranjuez when adding solutions with increasing amounts of $NH_4NO_3$, and this was attributed to the dominant response of one nitrophilous species. However, *A. linum-stellatum* and *L. echioides* density and aboveground biomass began to decline with N fertilization loads between 20 and 50 kg N $ha^{-1}$ $y^{-1}$ (Ochoa-Hueso and Manrique 2010). In this study, we attribute the reduced number of individuals with increased N fertilization to reduced germination rather than to greater plant mortality caused by alternative mechanisms, for example N burning, because we never saw dead plants, only a smaller number of individuals. Other mechanisms such as self-thinning are also discarded as plant density did not frequently reach values above 1 individual per 30 $cm^2$, which is very low given the really small size of these annual plants.

The significant effect of N fertilization on *A. linum-stellatum* RNE can be explained as a result of shrubs playing a slightly (but not completely) ameliorating effect against the negative consequences of increasing N loads, which could be linked to a higher abundance of other organisms under shrubs (such as mosses or microbes) actively taking up part of the N supplied (Ochoa-Hueso and others 2011; Gundale and others 2011). With regard to the independent effect of micro-site, it seems evident that *A. linum-stellatum* was clearly facilitated by the protective effect of shrubs against water stress and solar irradiance in terms of plant establishment, that is, there were more individuals and more biomass per area under shrubs (data not shown). However, individuals under shrubs were smaller, both aboveground and belowground, and with a higher proportion of belowground biomass, suggesting competition for soil resources. For this reason, we suggest that the low plant density of *A. linum-stellatum* in the interspaces must be conditioned by, at least, N deposition (including N fertilization treatments and N deposition background), water availability and light stress. In contrast, *L. echioides* was never present under shrubs, showing that the positive effects provided by woody plants are species-specific (Lortie and Turkington 2008).

*Asterolinon linum-stellatum* root growth was also reduced by N fertilization and this response could be explained by: (1) the functional equilibrium model, which predicts that N addition would reduce allocation to roots, (2) the putative role of direct soil N toxicity, or (3) both. The significant decrease in root:shoot ratio points to the role of the functional response, although the role of soil toxicity cannot be discarded. Briefly explained, amelioration of nutrient (N) availability increases allocation to aboveground structures in relation to roots, which has been related to shifts from belowground competition for nutrients to aboveground competition for light (Agren and Franklin 2003; Xia and Wan 2008).

To explain the role of soil $NO_3^-$-N and $NH_4^+$-N on the plant response to simulated N deposition we suggest the following mechanism: as the dominant mineral N form in semiarid shrublands, $NO_3^-$-N is usually positively correlated with plant production in natural conditions. Fertilization with $NH_4NO_3$ can, however, affect plant productivity and traits in two essential ways; that is, the oxidized form can slightly contribute to increase plant productivity (Figure 7), but only to a certain extent given that plants are primarily limited by water and other nutrients (mainly P), whereas the reduced form has a negative effect that reduces plant germination and productivity (Table 3), possibly to a greater extent than the positive effect exerted by increased $NO_3^-$-N. The use of $NO_3^-$-N and $NH_4^+$-N as covariates would, therefore, eliminate the confounding effects of pre-existing superimposed gradients. Figure 7 illustrates this type of response, showing the positive relationship between soil $NO_3^-$-N and *L. echioides* density independently for each N fertilization treatment (Figure 7). Whereas in the control treatments 12 ppm $NO_3^-$-N are enough to reach the highest plant density, in the

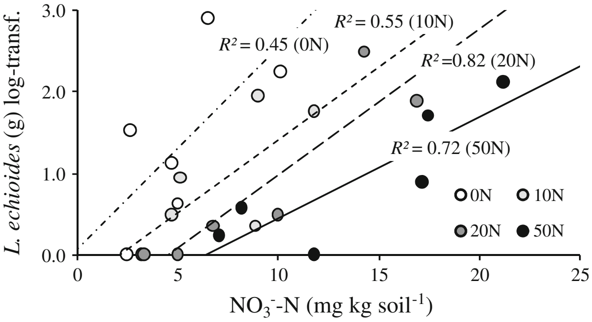

**Figure 7.** Relationship between *Limonium echioides* biomass production (log-transformed) and soil nitrate per N treatment in winter 2008/09. $N = 6$.

highest N load treatment 25 ppm are not even enough to reach similar density values (Figure 7).

Nitrogen fertilization also affected tissue chemistry in expected ways and independently of other superimposed gradients. Tissue N increased and C:N ratio decreased, both in *A. linum-stellatum* and *L. echioides*, whereas there was no significant effect on the analyzed shrub species; that is, shrubs are expected to be less responsive to N deposition than the annual plant community because of their longer lifespans, during which they are exposed to many varying environmental factors, and the presence of longer and deeper roots, giving them access to soil resources inaccessible to annual plants.

Several studies have been conducted to disentangle the main effects of N fertilization on microbial communities and, specifically, on symbiotic mycorrhizae, with most of them showing that higher N fertilization rates are associated with a decrease of root colonization (Egerton-Warburton and others 2007) and a reduced number of mycorrhizal fungi species (Santos and others 2006). Soil N enrichment might reinforce plant P deficiency (Egerton-Warburton and others 2007) and, in this context, Vitousek and others (2010) stated that many ecosystems are now poised to be near the point where N and P are equally limiting to the growth of plants because of N deposition. Egerton-Warburton and others (Egerton-Warburton and others 2007) also demonstrated that the AMF community structure appears to be controlled by soil N:P supply ratio, which is assumed to be very high in our highly P-limited soils (soils are frequently less than 1 ppm of extractable P). Thus, mycorrhizae might be helping to overcome the problem of increased N deposition and the related induced nutrient (N to P) imbalance, whereas the ability of AMF to respond to elevated N depends on the potential soil inoculums (quantity and species composition of the soil microbial community) and also on mycorrhizal dependency of each plant species (van der Heijden and others 1998). Thus, with a healthy and diverse soil inoculum, plants could exploit other resources (mainly P) to avoid excessive limitation. Our data suggest that plant responses to N deposition in terms of mycorrhizal associations could be highly dependent on the different potential inoculums found in the interspaces and under shrub cover (Martínez-García and others 2011), although the significance of the effect seems to be $NH_4^+$-dependent. The response of *A. linum-stellatum* plants under shrubs is toward increased root colonization with N addition, suggesting that P uptake by mycorrhizae might help to overcome increasing negative effects associated with increasing N loads found in the interspaces. This is also supported by the significant relationship between aboveground biomass and % arbuscules. Whether this response is associated with an alleviated N to P imbalance requires further analysis, but it is a plausible hypothesis. Indeed, Johnson and others (2003) pointed out that under P-limiting conditions, the investment of a host-plant in AMF should be strengthened to maintain the uptake of plant-limiting nutrients. In contrast, AMF root colonization in *L. echioides* did not significantly vary with N addition although a clear trend toward a reduction in root colonization was found (data not shown) and this reduction contrasted with the reduction of individuals in the interspaces, where potential soil inoculum quality could be lower. We have not found, however, any significant effect or trend when analyzing arbuscules, where the exchange of C and nutrients takes place. Thus we cannot be sure if the responses found for *A. linum-stellatum* are related to an increase of mycorrhizal fungi biomass after N enrichment (Wilson and others Wilson and others 2009) or if it has a concurrent functional implication. However, the coupling between plant and mycorrhizal responses points to the latter as a successful explanation. Furthermore, it is possible that N fertilization and the induced N to P imbalance are not only altering root colonization but also mycorrhizal species composition, shifting the community to one dominated by more nitrophilous species (Egerton-Warburton and others Egerton-Warburton and others 2007). For instance, N enrichment can reduce larger-spored mycorrhizal species with a concomitant proliferation of small-spored *Glomus* species, associated with a reduction in species richness, diversity and abundance of mycorrhizal species in California sites receiving 11–20 kg N $ha^{-1}$ $y^{-1}$ (Egerton-Warburton and Allen 2000). Yoshida and Allen (2001) also suggested that mycorrhizae are more important in controlling plant growth in native non-eutrophic coastal sage scrub soils dominated by $NH_4^+$-N rather than by $NO_3^-$-N. This supports the importance of soil $NH_4^+$-N in determining mycorrhizal responses to N deposition (Yoshida and Allen 2001), whereas $NO_3^-$-N seems to be more important in plant growth responses under low quality inoculums (in terms of low diversity and low density) conditions. Thus, not only aboveground but also belowground structure and functioning of arid and semiarid Mediterranean ecosystems could already be at risk of being significantly altered by N deposition. In our study, $NH_4^+$-N, other nutrients, and soil texture arose as important factors for natural AMF root colonization, supporting the importance of a wide range of soil factors and the aboveground environment for mycorrhizal colonization (Eschen and others Eschen and others 2009).

The reduction of shrub cover is also an expected response after crossing critical N loads (Vourlitis and Pasquini 2009), which are defined as a ''quantitative estimate of an exposure to one or more pollutants below which significant harmful effects on specified sensitive elements of the environment do not occur according to present knowledge'' (Nilsson and Greenfelt 1988). This seems to be the case for rosemary shrublands, whose critical load is predicted to be between 6.1 and 16.1 kg N $ha^{-1}$ $y^{-1}$, an estimation based on the fact that 10 kg N $ha^{-1}$ $y^{-1}$ plus the background N deposition (6.1 kg N $ha^{-1}$ $y^{-1}$) caused a decrease in rosemary twig growth. This value (16.1 kg N $ha^{-1}$ $y^{-1}$) is slightly higher than the mean N deposition value predicted for the Mediterranean Basin by 2050 (−12 kg N $ha^{-1}$ $y^{-1}$; Phoenix and others 2006). Padgett and Allen (1999) attributed this negative effect on native shrubs to a low capacity of plants to regulate their growth when N is supplied in excess of demand, leading to a more pronounced water stress during the dry season. Witkowski (1989b) also found increased litter production and P return in response to N addition during seasonal litter fall peak (late-spring to mid-summer), interpreted as indicative of increased water stress due to enhanced shoot growth. It seems likely that, even if rosemary shrubs were positively responding to N treatments in the first year of fertilization (data not shown), after longer exposures (possibly causing induced water stress) the effects became negative. Contrary to our findings, Sardans and others (2005) found little effects of N (500 kg N $ha^{-1}$ for 3 years) fertilization on rosemary shrubs from a post-fire calcareous shrubland in comparison with P (250 kg P $ha^{-1}$ for 3 years) additions, which was attributed to high levels of soil organic matter and mineral N. Another explanation for decreased shrub cover in N fertilized plots could be related to an increasing herbivore pressure (mainly rabbits) as a consequence of improved food quality in terms of N content. However, our tissue N data do not support this hypothesis, although we effectively observed evidence of browsing by rabbits in our experimental plots. It seems likely that this effect of simulated N deposition on shrubs, taken together with the reduction of the herbaceous cover in the interspaces, could worsen the situation for certain annual plant species exposed to increased N inputs. Shrub encroachment in semiarid sites worldwide is an example of how asymmetric and symmetric plant–plant interactions will be altered in the future in the context of the global change and altered environmental gradients (Maestre and others 2009).

## CONCLUSIONS

We conclude that vascular plant density, growth, tissue chemistry, and mycorrhizal colonization, were altered after 1.5 years of simulated N deposition, highlighting the high sensitivity of this type of semiarid Mediterranean ecosystems to human activities and, particularly, to air pollution. Specifically, two native annuals (and possibly the whole community, not studied here) were negatively affected in terms of density after accounting for the confounding effects of soil $NO_3^-$-N (the main form of inorganic N in soils of the study site), $NH_4^+$-N and pH. However, under shrub cover one of these species (*A. linum-stellatum*) was able to partially overcome the negative effects of either soil N toxicity or nutritional (N to P) imbalance. Shrub cover was also reduced after the addition of 20 kg N $ha^{-1}$ $y^{-1}$ over the background; whether these negative consequences on shrubs are related to an induced water stress, herbivore attack, or soil N toxicity is not known. However, if any of them is proven, then important consequences for native shrublands will derive from excessive anthropogenic inputs of N. Finally, the effects of N deposition on other response variables such as total productivity and C storage capacity of the plant community and the ability of nitrophilous and invasive plants to displace those of natural communities are still to be addressed from this ongoing experiment and also remain to be investigated in other areas with Mediterranean climate to understand the general functioning of semiarid Mediterranean ecosystems suffering chronic N deposition.

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